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Identification of a Novel Two-Peptide Lantibiotic, Lichenicidin, following Rational Genome Mining for LanM Proteins[▽]

Máire Begley,[1,2,3] Paul D. Cotter,[1,3]* Colin Hill,[1,2]* and R. Paul Ross[2,3]

Department of Microbiology, University College Cork, Cork, Ireland[1]; Alimentary Pharmabiotic Centre, University College Cork, Cork, Ireland[2]; and Teagasc, Dairy Products Research Centre, Moorepark, Fermoy, Cork, Ireland[3]

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Lantibiotics are ribosomally synthesized peptide antimicrobials which contain considerable posttranslational modifications. Given their usually broad host range and their highly stable structures, there have been renewed attempts to identify and characterize novel members of the lantibiotic family in recent years. The increasing availability of bacterial genome sequences means that in addition to traditional microbiological approaches, in silico screening strategies may now be employed to the same end. Taking advantage of the highly conserved nature of lantibiotic biosynthetic enzymes, we screened publicly available microbial genome sequences for genes encoding LanM proteins, which are required for the posttranslational modification of type 2 lantibiotics. By using this approach, 89 LanM homologs, including 61 in strains not known to be lantibiotic producers, were identified. Of these strains, five (*Streptococcus pneumoniae* SP23-BS72, *Bacillus licheniformis* ATCC 14580, *Anabaena variabilis* ATCC 29413, *Geobacillus thermodenitrificans* NG80-2, and *Herpetosiphon aurantiacus* ATCC 23779) were subjected to a more detailed bioinformatic analysis. Four of the strains possessed genes potentially encoding a structural peptide in close proximity to the *lanM* determinants, while two, *S. pneumoniae* SP23-BS72 and *B. licheniformis* ATCC 14580, possess two potential structural genes. The *B. licheniformis* strain was selected for a proof-of-concept exercise, which established that a two-peptide lantibiotic, lichenicidin, which exhibits antimicrobial activity against all *Listeria monocytogenes*, methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant enterococcus strains tested, was indeed produced, thereby confirming the benefits of such a bioinformatic approach when screening for novel lantibiotic producers.

Bacteriocins are microbially produced, ribosomally synthesized peptides that have a bactericidal or bacteriostatic effect on other species. One of the two major classes of bacteriocins are lantibiotics (lanthionine-containing antibiotics) and are distinguished by the cross-linking of cysteine to either dehydroalanine or dehydrobutyrine (resulting from the dehydration of hydroxyl amino acids) to form lanthionine and/or methyllanthionine residues. Other unusual posttranslationally modified amino acids including unlinked dehydroalanines and dehydrobutyrines can also be present (reviewed in references 7, 11, 15, and 19). The lantibiotics can themselves be subdivided on the basis of the nature of the enzymes responsible for these characteristic modifications. Type 1 lantibiotics (such as nisin, subtilin, and epidermin) are modified by a dual-enzyme system generically referred to as LanBC, while type 2 lantibiotics (such as lacticin 481, mersacidin, lacticin 3147, and cinnamycin) are modified by LanM proteins (33, 50). Lantibiotics have been the focus of extensive research in recent years, since it was established that many of them exhibit broad-range activity against a number of clinically relevant pathogens (6, 18, 24, 31, 41). At least some lantibiotics are active at single-nanomolar concentrations through a dual mechanism of action, which is facilitated by binding to lipid II, the "Achilles heel" of the gram-positive cell wall and a target of a number of clinically relevant antibiotics (4, 5, 48, 49). It is unsurprising that numerous screening strategies have taken place with a view to identifying novel lantibiotics with desirable properties such as enhanced potency, target specificity, or physicochemical properties. As with producers of antimicrobials in general, lantibiotic-producing strains have traditionally been screened by functional assays based on the inhibition of specific target spoilage or pathogenic microbes. In addition to being time-consuming, a lack of precise knowledge with respect to optimal lantibiotic-producing conditions (e.g., pH, incubation temperature, time of incubation, carbohydrate source, and temporal expression, etc.) and the use of a limited number of indicator strains can result in producing strains being overlooked. As the number of bacterial genome sequences available in public databases is increasing rapidly, it is likely that a postgenomic approach to identify novel bacteriocins may prove to be an attractive alternative.

In the current communication, we describe computational analyses employed to search sequenced bacterial genomes for novel type 2 lantibiotics. Of the putative LanM-encoding genes identified, 61 are in strains not previously reported to be producers of lantibiotics. Five strains that were subjected to closer in silico analysis revealed further evidence of the potential for the production of lantibiotic-like peptides, and one, *Bacillus licheniformis* ATCC 14580, through a combination of bioinformatic analyses, antimicrobial assays, mass spectrometry, and high-performance liquid chromatography (HPLC) analyses, was confirmed to be the producer of a broad-spectrum two-peptide lantibiotic, lichenicidin.

* Corresponding author. Mailing address: Department of Microbiology, University College Cork, Cork, Ireland. Phone for Paul D. Cotter: 353-25-42694. Fax: 353-25-42340. E-mail: paul.cotter@teagasc.ie. Phone for Colin Hill: 353-21-4901373. Fax: 353-21-4903101. E-mail: c.hill@ucc.ie.

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MATERIALS AND METHODS

Media, chemicals, and growth conditions. *B. licheniformis* ATCC 14580 (isogenic to DSM13) was obtained from the American Type Culture Collection (ATCC) and was routinely grown in either brain heart infusion broth or Luria-Bertani (LB) broth at 37°C. *Listeria monocytogenes* (EGDe, LO28, 10403s, CD147, CD1032, CD1028, CD1066, CD243, CD98, DPC4606, CD749, CD238, CD878, DPC4609, CD1059, and CD246), *Listeria innocua*, *Bacillus cereus*, *Bacillus halodurans* C-125, *Bacillus subtilis*, *Streptococcus mutans* 257, *Streptococcus pneumoniae* TIGR4, *Staphylococcus aureus* (ST285, ST525, ST523, ST533, ST355, ST530, ST535, ST544, ST522, and ST353), *Enterococcus faecium* (EC533, EC667, EC548, EC538, EC587, EC676, EC300, and EC89), and *Enterococcus faecalis* (EC618 and EC655) strains were grown in brain heart infusion broth at 37°C. *Lactococcus lactis* (HP, DPC3147, 104, 497, AM2, and MG1363) strains were grown in GM17 at 30°C. All media were purchased from Oxoid.

Sequence analyses. Bioinformatic analyses to identify novel lantibiotics were performed using the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). PSI-BLAST searches (3) were performed with the lacticin 3147 modification enzyme LtnM1 (GenBank accession number NP_047321) as a driver sequence. All significant hits were examined. In selected cases, the corresponding bacterial genomes were accessed through the genome database, and the regions containing the genes of interest were located. These genomes were also analyzed by BAGEL, a Web-based bacteriocin genome-mining tool (14). Where necessary, protein sequences were further analyzed using various Web-based programs including those located at http://psort.nibb.ac.jp/, http://www.sbc.su.se/~miklos/DAS/maindas.html, http://.cbs.dtu.dk/services/TMHMM/, and http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml. Protein alignments were carried out using CLUSTAL W (http://www.ebi.ac.uk/clustalw/).

Inhibition spectrum. For well diffusion assays, 20 ml of agar was seeded with 50 μl of a culture of the test strain (approximately 1×10^6 CFU/ml) grown overnight, and when set, 8-mm holes were bored with the end of a sterile glass pipette. Cell-free supernatant or crude bacteriocin preparation was diluted in Ringer's solution, and 50 μl of each dilution was added to separate wells. Plates were incubated overnight at a temperature suitable for the growth of the test strain. For deferred antagonism assays, 10 μl of a *B. licheniformis* ATCC 14580 culture grown overnight was spotted onto 20 ml of LB agar and allowed to grow at 37°C overnight. Cells were killed by a 20-min exposure to UV light, and plates were then overlaid with soft agar (0.75% agar) containing 50 μl (1×10^6 cells) of a culture of the test strain grown overnight. Plates were incubated overnight at a temperature suitable for the growth of the test strain.

Generation of a crude lantibiotic preparation. A method similar to that used previously for the purification of lacticin 3147 (9) was used to obtain a preparation of lantibiotic from whole *B. licheniformis* ATCC 14580 cells. A culture of *B. licheniformis* ATCC 14580 grown overnight was inoculated (1%) into 1 liter of LB broth and incubated at 37°C overnight (approximately 16 to 18 h) with shaking. Cells were harvested by centrifugation at ~7,500 × *g* for 20 min and resuspended in 250 ml of 70% propan-2-ol (adjusted to pH 2 through the addition of concentrated HCl). After 5 h of stirring at 4°C, cell debris was removed by centrifugation, and the supernatant was reduced to approximately 60 ml by rotary evaporation, freeze-dried, and resuspended in 3 ml of 2.5 mM sodium phosphate buffer. Activity was examined by well diffusion assays using *L. lactis* HP as the indicator strain.

Purification of lichenicidin. *B. licheniformis* ATCC 14580 was grown overnight at 37°C with shaking in LB broth, after which it was inoculated (1%) into tryptone-yeast extract-glucose in conical flasks and reincubated for a further 18 h at 37°C with shaking. The culture was centrifuged at ~7,500 × *g* for 20 min, and the supernatant was removed and discarded. Cell pellets were resuspended in 300 ml of 70% isopropanol–0.1% trifluoroacetic acid and stirred at room temperature for approximately 3 h. Samples were centrifuged (~7,500 × *g* for 20 min), and supernatants were retained. The isopropanol was evaporated using a rotary evaporator (Buchi) before application onto a Phenomenex SPE C_{18} column preequilibrated with methanol and water. The column was washed with 120 ml of 30% ethanol, and bacteriocin was eluted in 100 ml 70% propan-2-ol (pH 2). Ten milliliters was concentrated to 1 ml by rotary evaporation and applied onto a Phenomenex C_{12} reverse-phase HPLC column previously equilibrated with 25% propan-2-ol–0.1% trifluoroacetic acid. The column was developed in a gradient of 35% to 65% propan-2-ol at a flow rate of 1.2 ml/min.

Mass spectrometric analysis. For colony mass spectrometry (CMS), colonies were mixed with 50 μl of 70% isopropanol (pH 2), vortexed, and centrifuged at 14,000 × *g* for 2 min. The supernatant was removed for analysis. The mass of peptides present in this supernatant or in HPLC fractions was determined with an Axima CFR Plus matrix-assisted laser desorption ionization–time of flight mass spectrometer (Shimadzu Biotech, Manchester, United Kingdom) as previously described (25, 29).

RESULTS

Identification of LanM homologs in sequenced genomes. A strategy was employed to identify novel type 2 lantibiotics using an in silico approach that takes advantage of the highly conserved nature of the associated lanthionine synthetase, generically termed LanM. A representative protein, LtnM1, one of two such enzymes responsible for the modification of the two-peptide lantibiotic lacticin 3147, was selected as the driver for this screen, which involved a PSI-BLAST (3) search of the NCBI database (http://www.ncbi.nlm.nih.gov). All results with significant E values were examined. It was noted that hits below the cutoff point included known non-LanM proteins, such as type 1-associated LanC homologs, which are known to exhibit low-level sequence identity to LanM proteins (43). In total, 89 proteins with homology to LtnM1 were apparent (Table 1). These included proteins previously associated with the production of the lantibiotics lacticin 3147; staphylococcin C55; mersacidin; michigainin; haloduracin; nukacin ISK-1; cytolysin; streptococcin A-FF22; mutacins II and K8; Smb; BHT-A; salivaricins A, A1, A2, 9, and B; macedocin; butyrivibriocin OR79A; ruminococcin A; lactocin S; and cinnamycin as well as 61 proteins predicted to be produced by strains not previously identified as being lantibiotic producers (Table 1). While a number of the strains are representatives of species previously associated with lantibiotic production, many are from species, genera, and even phyla for which the biosynthesis of these antimicrobials has never been reported or screened for.

Specific examination of five potential lantibiotic gene clusters. Of the potential producers, five were selected for a more detailed bioinformatics analysis, including *Streptococcus pneumoniae* SP23-BS72 (CGSSp23BS72_03618), *Bacillus licheniformis* ATCC 14580 (Bli04128), *Anabaena variabilis* NCPPB (Ava_B0238), *Geobacillus thermodenitrificans* NG80-2 (GTNG_2062), and *Herpetosiphon aurantiacus* ATCC 23779 (Haur_1868). This involved an analysis of regions flanking LanM determinants for other open reading frames (ORFs) potentially involved in the biosynthesis of, or immunity to, lantibiotics (taking advantage of the fact that lantibiotic genes are often organized into one or more adjacent operons). More specifically, a screen for genes potentially encoding structural peptides (designated LanA) was undertaken. These peptides consist of a cysteineless leader region that terminates with a "GG" leader cleavage site (GG, GA, or AG) and a structural propeptide containing a number of cysteine, threonine, and serine residues that can be subsequently modified by LanM. While homology can exist between individual unmodified LanA peptides and is the basis of one approach to the classification of lantibiotics (10), LanA peptides that are completely unlike any other known peptide have been, and continue to be, identified. We also screened for other relevant genes encoding proteins involved in modification (*lanB*, *lanC*, *lanM*, *lanD*, and *lanJ*), processing (*lanP* and *lanT*), transport (*lanT* and *lanH*), immunity (*lanI* and *lanFEG*), and regulation (*lanR*, *lanK*, *lanQ*, and *lanX*).

Streptococcus pneumoniae SP23-BS72, a clinical pneumonia-associated isolate (42), possesses a number of additional po-

TABLE 1. LtnM1 homologs identified by a PSI-BLAST search of the NCBI database[a]

Strain	LanM homolog	GenBank accession no.	Identity (%)	Similarity (%)	Associated lantibiotic
Lactococcus lactis DPC3147 pMRC01	LtnM1	NP_047321.1	100	100	Lacticin 3147
Staphylococcus aureus pETB	SacM1	NP_478385.1/AAD47013.1	44	64	Staphylococcin C55
"*Anaerocellum thermophilum*" DSM6725	Athe_1107	YP_00257298101	27	44	Unknown
Streptococcus pneumoniae SP23-BS72	CGSSp23BS72_03618	ZP_01834975.1	28	44	Unknown
Bacillus licheniformis ATCC 14580	BliLi04128	YP_081205.1	24	44	Unknown
"*Microcoleus chthonoplastes*" PCC7420	MC7420_5497	YP_002621143.1	24	42	Unknown
Bacillus sp. strain HIL-Y85/54728	MrsM	CAB60261.1	25	44	Mersacidin
Clavibacter michiganensis NCPPB 382	ClvM	YP_001222710.1	25	44	Michingainin
Bacillus halodurans C-125	BhaM1	NP_241321.1	24	44	Haloduracin
Anabaena variabilis ATCC 29413 plasmid B	Ava_B0238	YP_320138.1	24	43	Unknown
Geobacillus thermodenitrificans NG80–2	GTNG_2062	YP_001126159.1	23	43	Unknown
Geobacillus sp. strain G11MC16	G11MC16DRAFT_3401	ZP_03149642.1	23	43	Unknown
Herpetosiphon aurantiacus ATCC 23779	Haur_1868	YP_001544639.1	23	41	Unknown
Bacillus cereus Q1	BCQ_4956	YP_002532646.1	23	43	Unknown
Streptococcus pneumonia ATCC 700669	SPN23F_12700	YP_002511204.1	23	43	Unknown
Nostoc punctiforme PCC 73102	Npun02007880	ZP_00106276.1	22	41	Unknown
Blautia hansenii DSM20583	BLAHAN_00750	ZP_03546987.1	23	42	Unknown
Cyanothece sp. strain PCC7425	Cyan7425_5238	ZP_002485891.1	24	42	Unknown
Myxococcus xanthus DK 1622	MXAN_2857	YP_631068.1	23	40	Unknown
Cyanothece sp. strain PCC7425	Cyan7425_2897	YP_002483601.1	*23*	*41*	Unknown
Streptomyces griseus subsp. *griseus* NBRC13350	SGR_4809	YP_001826321.1	23	43	Unknown
Stigmatella aurantiaca DW4/3-1	STIAU_2781	ZP_01460524.1	22	41	Unknown
Corynebacterium diptheriae NCTC 13129	DIP0753	NP_939126.1	21	41	Unknown
Staphylococcus warneri	NukM	NP_940773.1	24	42	Nukacin ISK-1
Enterococcus faecalis pAD1	CylM	AAA62650.1	23	42	Cytolysin
Nostoc sp. strain PCC 7120	all2025	NP_486065.1	22	41	Unknown
Synechococcus sp. strain RS9916	RS9916_34542	ZP_01470939.1	21	40	Unknown
Lactococcus lactis DR2	LctM	P37609	23	42	Unknown
Clostridium scindens ATCC 35704	CLOSCI_03314	ZP_02433053.1	23	45	Unknown
Myxococcus xanthus DK 1622	MXAN_6388	YP_634512.1	21	41	Unknown
Nostoc punctiforme PCC 73102	Npun02004035	ZP_00110236.1	22	40	Unknown
Streptococcus pyogenes	ScnM	AAB92602.1	22	42	Streptococcin A-FF22
Coxiella burnetii Dugway 5J108-111	COXBU7E912_1243	YP_001424603.1	21	40	Unknown
Prochlorococcus marinus MIT 9303	P9303_21071	YP_001018107.1	22	40	Unknown
Bacillus licheniformis ATCC14580	BLi04126	YP_081203.2	22	43	Unknown
Coxiella burnetii MSU Goat Q177	A35_A1154	ZP_01946732.1	21	40	Unknown
Bacillus halodurans C-125	BH0452	NP_241318.1	25	45	Haloduracin
Salinispora arenicola CNS-205	Sare_0349	YP_001535270.1	24	47	Unknown
Stenotrophomonas sp. strain SKA14	SSKA14_3703	YP_002708103.1	21	37	Unknown
Nostoc punctiforme PCC 73102	Npun02004880	ZP_00108421.2	21	40	Unknown
Mycobacterium marinum M	MMAR_0918	YP_001849230.1	20	40	Unknown
Streptococcus mutans	MukM	ABK59358.1	23	42	Mutacin K8
Prochlorococcus marinus MIT 9313	PMT0250	NP_894083.1	22	39	Unknown
Coxiella burnetti CbuGQ212	CbuG_0738	YP_002303280.1	21	40	Unknown
Streptococcus salivarius	SivM	ABI54435.1	21	42	Salivaricin 9
Bifidobacterium angulatum DSM20098	BIFANG_01012	ZP_03448967.1	22	39	Unknown
Lactococcus lactis DPC3147 pMRC01	LtnM2	NP_047323.1	23	40	Lacticin 3147
Streptococcus salivarius K12	SboM	ABI63640.1	22	42	Salivaricin B
Coxiella burnetii Dugway 5J108-111	COXBU7E912_1359	YP_001424711.1	21	41	Unknown
Nostoc punctiforme PCC 73102	Npun02002371	ZP_00110570.1	23	41	Unknown
Herpetosiphon aurantiacus ATCC 23779	Haur_3738	YP_001546502.1	27	45	Unknown
Cyanothece sp. strain PCC7425	Cyan7425_3979	YP_002484655.1	22	40	Unknown
Streptococcus macedonicus	McdM	ABI30229.1	23	41	Macedonicin
Streptococcus pneumoniae CDC0288-04	SpneCD_010100008282	ZP_02716217.1	22	40	Unknown
Sinorhizobium medicae WSM419 pSMED02	Smed_6089	YP_001314664.1	22	39	Unknown
Streptococcus pneumoniae TIGR4	SP_1950	NP_346378.1	22	40	Unknown
Streptococcus pneumoniae R6/D39	CylM-like protein	NP_359359.1	22	40	Unknown
Streptococcus pneumoniae CDC1873-00	SpneCDC1_010100008806	ZP_02709201.1	22	40	Unknown
Streptococcus equinus	BovM	ACA51935.1	24	42	Unknown
Butyrivibrio fibrisolvens	OR79 M	AAC19356.1	22	42	Butyrivibriocin OR79A
Cyanothece sp. strain PCC7425	Cyan7425_3047	YP_002483742 1.1	21	38	Unknown
Clostridium beijerinckii NCIMB 8052	Cbei_4586	YP_001311650.1	24	45	Unknown
Stigmatella aurantiaca DW4/3-1	STIAU_1974	ZP_01462725.1	24	40	Unknown
Clostridium perfringens B ATCC 3626	CperB13066	ZP_02636763.1	22	41	Unknown
Clostridium hylemonae DSM15053	CLOHYLEM_07236	ZP_03780146.1	20	40	Unknown
Azorhizobium caulinodans ORS 571	AZC_3208	YP_001526124.1	21	38	Unknown
Saccharopolyspora erythraea NRRL 2338	SACE_4389	YP_001106583.1	23	43	Unknown
Streptococcus ratti	BhtM1	AAZ76597.1	24	41	BHT-A
Ruminococcus gnavus ATCC 29149	RUMGNA_01473	ZP_02040709.1	21	41	Putative ruminococcin
Ruminococcus gnavus	RumM	CAB93674.2	22	40	Ruminococcin A
Streptococcus pneumoniae SP19-BS75	CGSSp19BS75_00831	ZP_01833492.1	24	46	Unknown
Lactobacillus sakei	LasM	CAA91110.1	22	44	Lactocin S
Streptococcus mutans	SmbM1	BAD72771.1	27	42	Mutacin Smb
Staphylococcus aureus pETB	SacM2	NP_478387.1	23	40	Staphylococcin C55
Streptococcus pyogenes MGAS10750	MGAS10750_Spy1727	YP_603221.1	25	41	Salivaricin A1
Ruminococcus gnavus	RumM	AAK73192.1	21	40	Ruminococcin A (disrupted operon)
Corynebacterium matruchotti ATCC 33806	CORMATOL_02556	ZP_03711708.1	22	39	Unknown
Bifidobacterium longum DJO10A	Blon03001836	ZP_00206332.1	21	40	Unknown
Streptomyces cinnamoneus	CinM protein	CAD60521.1	23	39	Cinnamycin
Streptococcus salivarius	SalM	ABI63629.1	25	41	Salivaricin A2
Bifidobacterium longum DJO10A	BLD_1651	YP_001955594.1	21	40	Unknown
Clostridium perfringens D JGS 1721	CJD_0434	ZP_02952302.1	23	40	Unknown
Cyanothece sp. strain PCC8801	PCC8801_1979	YP_002372173.1	21	40	Unknown
Streptococcus salivarius	SalB	AAG32536.1	26	40	Salivaricin A
Streptococcus mutans	MutM	AAC38145.1	22	38	Mutacin II
Ruminococcus gnavus ATCC 29149	RUMGNA_01470	ZP_02040706.1	28	46	Unknown
Cyanothece sp. strain PCC8802	Cyan8802DRAFT_3943	ZP_03145546.1	21	40	Unknown
Corynebacterium matruchotti ATCC 33806	CORMATOL_02552	ZP_03711704.1	23	39	Unknown
Streptococcus suis 05ZYH33	SalBSSU05_0960	YP_001198326.1	24	40	Salivaricin-like

[a] Information was collated on 26 March 2009.

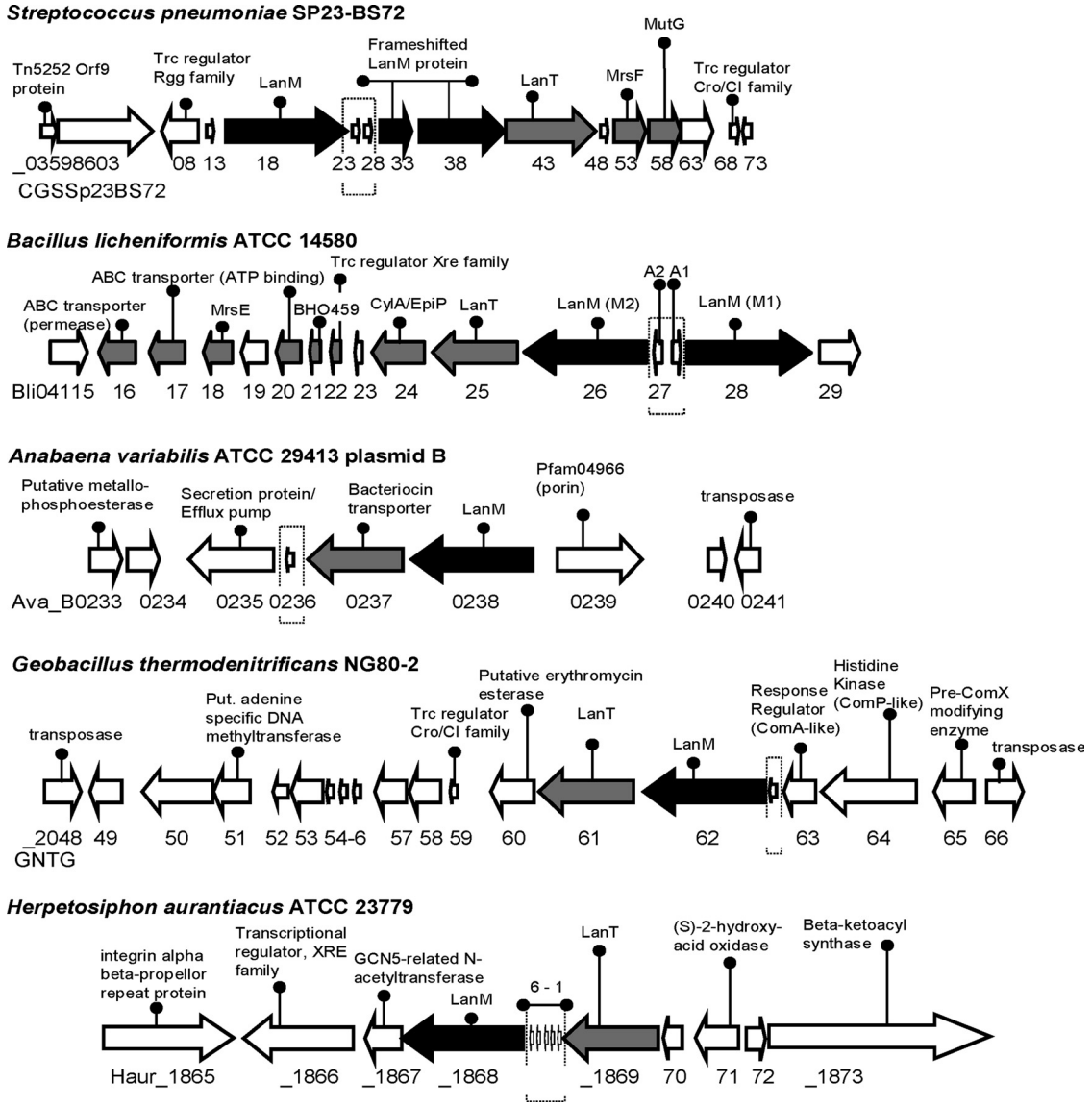

FIG. 1. Line diagram of predicted lantibiotic gene clusters. This diagram was drawn approximately to scale using data from genome sequences. Numbers refer to NCBI annotation numbers. LanM determinants are indicated in black. Genes encoding products predicted to resemble known lantibiotic-associated proteins are in gray. Putative (Put.) structural peptide determinants are boxed.

tentially lantibiotic-associated genes in close proximity to the putative LanM determinant (CGSSp23BS72_03618) (13). These include a second, apparently frameshifted, LanM determinant (CGSSp23BS72_03598633 and CGSSp23BS72_03598638, 26% identical to LtnM2 of lacticin 3147 and 28% identical to HalM2 of haloduracin, respectively) as well as genes encoding a putative LanT lantibiotic transporter (CGSSp23BS72_03598643, 39% identical to HalT of haloduracin) and LanFG ABC transporters, often associated with immunity (CGSSp23BS72_03598653 and CGSSp23BS72_03598658, 47% identical to MrsF of mersacidin and 25% identical to MutG of mutacin II, respectively) (Fig. 1). Significantly, two neighboring small ORFs (CGSSp23BS72_03598623 and CGSSp23BS72_03598628), which we designate PnmA1 and PnmA2, respectively, potentially encode the individual prepeptides of a two-peptide lantibiotic (Fig. 1 and 2). Two-peptide lantibiotics are active via the synergistic activity of two lanthionine-containing peptides and include lacticin 3147, produced by *Lactococcus lactis* DPC3147 (38); staphylococcin C55, produced by *Staphylococcus aureus* C55 (32); plantaricin W, produced by *Lactobacillus plantarum* (20); Smb, produced by *Streptococcus mutans* GS5 (51); BHTA, produced by *Streptococcus rattus* BHT (21); and haloduracin, produced by *Bacillus halodurans* C-125 (25, 29; for a review, see reference 26). As with other such peptides, PnmA1 and PnmA2 both contain possible leader regions, which end with GG or GA leader cleavage sites (Fig. 2A and B).

The second gene cluster studied is located on one of three plasmids found in *A. variabilis* ATCC 29413, a filamentous heterocyst-forming cyanobacterium. It is notable that although cyanobacteria are not known as lantibiotic producers, a number of *lanM* homologs listed in Table 1 are located in other

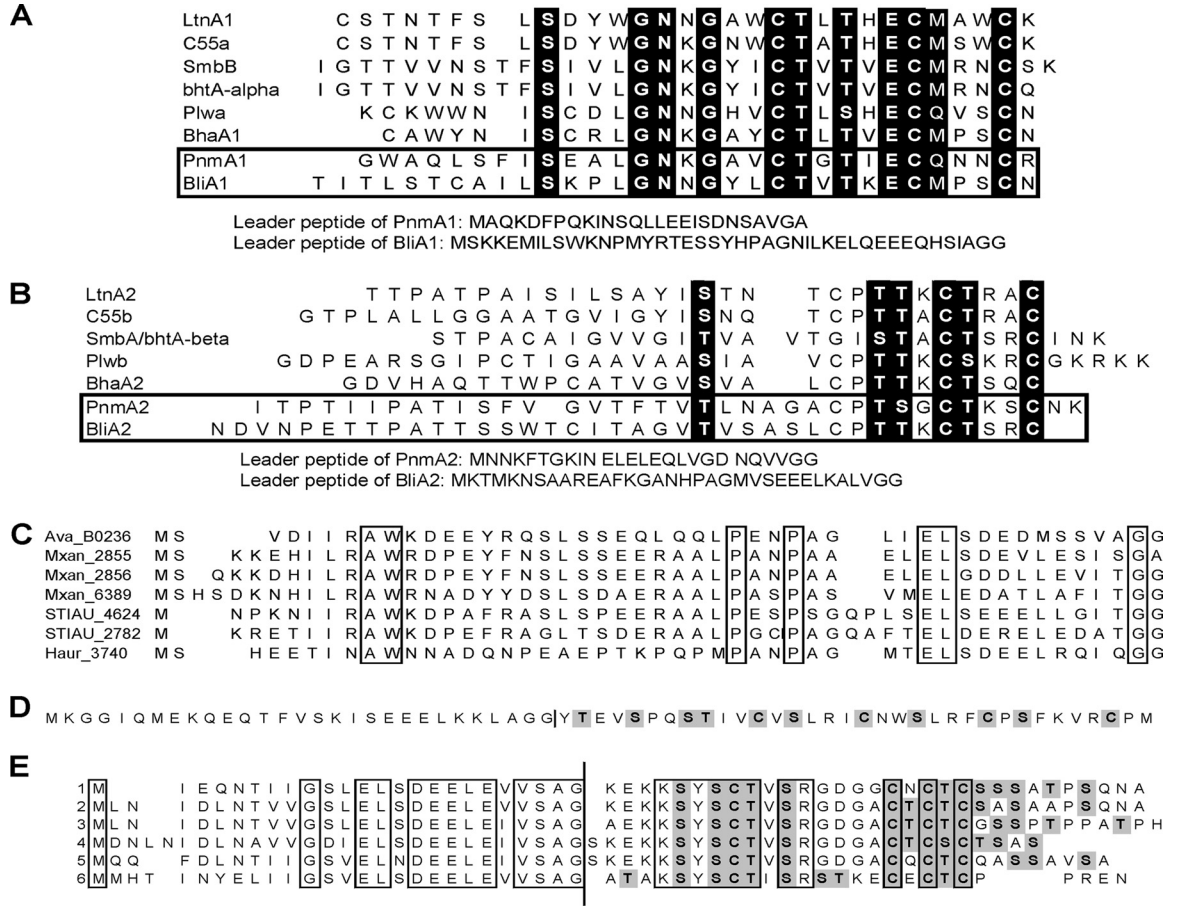

FIG. 2. Predicted structural peptides (unmodified form) identified as a consequence of an in silico screen for novel lantibiotics. (A) Alignment of the A1 structural propeptides PnmA1 and BliA1 with related peptides. Residues contained within a conserved motif are highlighted (black background and white font). (B) Alignment of the A2 structural propeptides PnmA2 and BliA2 with related peptides. Residues contained within a conserved motif are highlighted (black background and white font). (C) Alignment of the leader region of the putative *A. variabilis* ATCC 29413 structural peptide, Ava_B0236, with corresponding peptides predicted to be produced by *M. xanthus* DK 1622, *Stigmatella aurantiaca* DW4/3-1, and *Herpetosiphon aurantiacus* ATCC 23779. Completely conserved residues are boxed. (D) Lantibiotic peptide predicted to be produced by *G. thermodenitrificans* NG80-2. The predicted leader cleavage site is marked, and potentially modified residues have a gray background. (E) Alignment of six related, potential lantibiotic, peptides predicted to be produced by *Herpetosiphon aurantiacus* ATCC 23779. Completely conserved residues are boxed. The predicted leader cleavage site is marked, and potentially modified residues have a gray background. Alignments were performed using CLUSTAL W (http://www.ebi.ac.uk/clustalw/) in all cases.

representatives including *Nostoc*, *Synechococcus*, and *Prochlorococcus* species. With respect to the plasmid-located cluster in ATCC 29413 (Fig. 1), Ava_B0235 and Ava_B0238 are predicted to be a secretion protein and the membrane domain of an ABC transporter, respectively, while the product of Ava_b0236 is predicted to possess a number of lantibiotic propeptide-like features including a leader region devoid of cysteine residues with a GG leader cleavage motif (Fig. 2C) and a structural portion that contains seven potentially modified serine, threonine, or cysteine residues (not shown). However, possibly the most significant feature of this peptide is the fact that related peptides are encoded by ORFs located adjacent to the *lanM* homologs in *Myxococcus xanthus* DK1622 (MXAN_2855, MXAN_2856, and MXAN_6389 for *lanA* and MXAN_2857 and MXAN_6388 for *lanM*), *Stigmatella aurantiaca* DW4/3-1 (STIAU_2782 for *lanA* and STIAU_2781 for *lanM*), and *Herpetosiphon aurantiacus* ATCC 23779 (Haur_3740 for *lanA* and Haur_3738 for *lanM*). This resemblance is specifically within the proposed leader regions (Fig. 2C), which, in addition to facilitating export, possibly play a role in the recognition of the peptides by modification enzymes such as LanM enzymes during biosynthesis.

G. thermodenitrificans is a thermophilic bacillus isolated from a deep oil reservoir in Northern China (17). With respect to its putative lantibiotic-encoding gene cluster, a putative LanT determinant (GNTG_2061) as well as an unannotated possible structural peptide determinant (located between GNTG_2062 and GNTG_2063) are located in close proximity to the putative LanM-encoding gene (GNTG_2062) (Fig. 1). The putative structural peptide again contains a leader region, which lacks cysteine residues and ends with a GG motif preceding a peptide potentially containing as many as 11 modified residues (Fig. 2D). Response regulator, histidine kinase, and regulator determinants are also located in this region, but it is unclear if they contribute to lantibiotic production (Fig. 1).

The fourth cluster is located in *H. aurantiacus*. *H. aurantia-*

TABLE 2. In silico analyses of predicted proteins in the *B. licheniformis* ATCC 14580 bacteriocin cluster[a]

Protein (GenBank accession no.)	Size (amino acids)	Description	Closest homolog (GenBank accession no.)	Predicted function
BLi04116 (YP_093622)	313	COG1277; NosY permease component of an ABC-type transport system (6 TMS)	Hypothetical protein BsubsN3_04969 of *B. subtilis* (ZP_03594875)	Permease (ABC transporter, immunity)
BLi04117 (YP_093623)	300	COG1131; CcmA ATPase component of an ABC-type multidrug transport system	ABC transport-related protein of *Geobacillus* sp. strain Y412MC10 (ZP_03040055)	ATPase (ABC transporter, immunity)
BLi04118 (YP_093624)	249	6 TMS	Conserved hypothetical protein of *Geobacillus* sp. strain G11MC16 (ZP_03148320)	Immunity
BLi04119 (YP_093625)	218	5 TMS	No hits	No prediction
BLi04120 (YP_093626)	211	COG1131	ATP-binding protein of *Clostridium botulinum* E3 Alaska E43 (YP_001920999)	ATPase (ABC transporter, immunity)
BLi04121 (YP_093627)	95		BHO459 of *B. halodurans* C-125 (BAB04178)	No prediction
BLi04122 (YP_093628)	92	COG1476; predicted transcriptional regulators	BHO460 of *B. halodurans* C-125 (BAB04179)	Transcriptional regulator
BLi04123 (YP_093629)	62		Hypothetical protein BB14905_14460 of *Bacillus* sp. strain B14905	No prediction
BLi04124 (YP_093630)	443	COG1404; subtilisin-like serine proteases; Pfam00082, peptidase	CylA of *E. faecalis* (AAA62652)	Serine protease
BLi04125 (YP_093631)	718	COG2274; SunT ABC-type bacteriocin exporter	BHO451 of *B. halodurans* C-125 (BAB04170)	Transporter
BLi04126 (YP_093632)	1,036	COG4403; lantibiotic-modifying enzyme; Pfam05500, NisC domain	BH0452 of *B. halodurans* C-125 (BAB04171)	Modification enzyme
BLi04126b (not annotated)	72		BH0453 of *B. halodurans* C-125 (BAB04172)	Lantibiotic prepeptide
BLi04127 (YP_093633)	74		BH0454 of *B. halodurans* C-125 (BAB04173)	Lantibiotic prepeptide
BLi04128 (YP_093634)	1,052	COG4403; lantibiotic-modifying enzyme; Pfam05500, NisC domain	BH0455 of *B. halodurans* C-125 (BAB04174)	Modification enzyme

[a] Homology searches were performed on 26 March 2009.TMS, transmembrane segments as predicted by the DAS (http://www.sbc.su.se/~miklos/DAS/maindas.html) and TMHMM (http://.cbs.dtu.dk/services/TMHMM/) databases; COG, cluster of orthologous groups of proteins.

cus is a filamentous bacterium found most frequently in soil, freshwater, and sewage treatment plants. The strain with its genome sequenced, ATCC 23779, possesses two putative lantibiotic-encoding gene clusters, one of which, as noted above, contains a structural gene resembling that associated with *A. variabilis* ATCC 29413. The second such cluster encodes a putative LanM protein, which even more closely resembles LtnM1. Upon first examination, the corresponding genomic region lacks any other lantibiotic-associated features except for the presence of an ABC transporter determinant (Haur_1869) (Fig. 1). However, closer inspection reveals that between Haur_1868 and Haur_1869, one finds six previously unannotated and almost identical ORFs, all of which are predicted to code for peptides with a cysteine-free, AG-ending leader as well as a structural peptide with 12 to 14 potentially modified residues (Fig. 2E), which are the most likely target of LanM-catalyzed modification. While the presence of multiple structural peptide determinants was previously noted (e.g., macedocin has three such genes [34]), the existence of six such copies has not previously been reported.

Identification and sequence analysis of a bacteriocin-related cluster in *B. licheniformis* ATCC 14580. Of the five strains selected for closer inspection, *B. licheniformis* ATCC 14580 was the focus of particular attention due to the presence of two, nonframeshifted, LanM determinants and because the bacilli were noted to be producers of a number of potent lantibiotics. The fact that this strain is nonpathogenic and has already been the subject of research with a view to industrial applications (36) also suggested that a lantibiotic produced by this strain could potentially be clinically useful. The genetic organization of the lantibiotic-related cluster is shown in Fig. 1. ORFs BLi04116 to BLi04128, inclusive, are predicted to encode a two-peptide lantibiotic and associated proteins involved in modification, processing, transport, immunity, and regulation. The predicted products of flanking ORFs do not seem to be bacteriocin related (BLi04115 and BLi04129 are predicted to encode a ferrochetalase and a pectate lyase, respectively). The 14-gene cluster (an additional unannotated ORF was identified between BLi04126 and BLi04127 and was designated BLi04126b) was analyzed using various Web-based programs (listed in Materials and Methods), the results of which are summarized in Table 2. BLi04116 to BLi04118 encode the putative self-protection immunity genes *lanEFG*. BLi04122 encodes a putative regulator that shows significant homology (38% identity) to LtnR (GenBank accession number NP_047318), a transcriptional repressor of the immunity genes of

the two-peptide lantibiotic lacticin 3147. BLi04124 encodes a putative serine protease that shows significant homology to CylA (37% identity) of *Enterococcus faecalis* (accession number AAK67268) and EpiP of *S. aureus* (accession number YP_186702) (29% identity), involved in production of the lantibiotics cytolysin and epidermin, respectively (39, 40). BLi04125 encodes a potential transporter that is homologous to LtnT of *L. lactis* pMRCO1 (32% identity) (accession number CAB60262) (16) and MrsT (34% identity) (accession number NP_047322) (2) of *Bacillus* sp. strain HIL-Y85/54728, both of which contribute to the export and leader cleavage of lantibiotics (lacticin 3147 and mersacidin, respectively). The two LanM proteins BLi04126 and BLi04128 are 30% identical to each other but are more homologous to BH0452 and BH0453, respectively. BH0452 and BH0453 are the two modification enzymes associated with the biosynthesis of the lantibiotic haloduracin by *B. halodurans* (34% identity in both cases) (25, 29). The putative BLi04119 and BLi04123 proteins do not show homology to any proteins in the public databases, so computational analyses could not predict a function. BLi04127 and BLi04126b encode the predicted lantibiotic structural peptides, designated BliA1 and BliA2 (in unmodified form) or Bliα and Bliβ (modified), respectively. The presence of two structural peptide determinants, combined with the presence of two LanM determinants, suggests that, if expressed, this cluster of genes is responsible for the production of a two-peptide lantibiotic (26, 29). With respect to both BliA1 and BliA2, putative leader peptides with a double-glycine motif were identified, indicating the presence of leader peptides of 42 and 34 amino acids, respectively. With respect to the leaderless propeptides, BliA1 and BliA2 are most homologous to HalA1 and HalA2, the corresponding leaderless propeptides of haloduracin (40% and 54% identity, respectively). BliA1 and BliA2 were aligned with the structural peptides of the six related two-peptide lantibiotics and PnmA1 and PnmA2 for comparative purposes (Fig. 2A and B). As a result, it was apparent that a number of residues are conserved across all peptides. These residues include a number of Cys, Ser, and Thr residues that are known to be involved in bridge formation in the corresponding lacticin 3147 and plantaricin W (20, 27, 29) as well as a Glu in the A1/α peptides that is involved in lipid II binding. Other than these, there are conserved Gly/Asn residues in the A1/α peptides and a Thr (known to be unmodified in LtnA2) in the A2/β equivalents that may be involved in the other conserved function of all of these peptides, namely, the ability to interact with the corresponding "sister" peptide. Viewed in combination, it is apparent that BliA1/PnmA1 and BliA2/PnmA2 contain motifs that closely resemble those previously noted as being common to all related A1 (SxxxGNxGxxCTxTxECmxxC) and A2 (s/txxxxcps/tTxCs/txxC) peptides, respectively (9, 26) (Fig. 2).

Production of an antimicrobial substance by *B. licheniformis* ATCC 14580 and partial purification of lichenicidin. Deferred antagonism assays confirmed that *B. licheniformis* ATCC 14580 produces an antimicrobial compound (Fig. 3A). Additional assays established that all gram-positive strains tested were sensitive, including 10 *L. monocytogenes* strains; 10 methicillin-resistant *Staphylococcus aureus* strains; 10 vancomycin-resistant enterococci; selected *B. cereus*, *Streptococcus pneumoniae*, and *Streptococcus mutans* strains; and nonpathogenic species, including *L. innocua* and *L. lactis* (data not shown). Two producers of two-peptide lantibiotics (*L. lactis* DPC3147, producing lacticin 3147, and *B. halodurans* C-125, producing haloduracin) were also inhibited, establishing that the natural immunity mechanisms in these strains do not provide protection against lichenicidin. The antimicrobial appears to be cell associated, as cell-free supernatant from *B. licheniformis* ATCC 14580 cultures lacked antimicrobial activity when assessed by agar well diffusion assays. CMS analysis revealed the presence of two peaks with masses of 3,020.4 and 3,251.5 Da (corresponding to the M + H forms of the peptides) on the cell surface (Fig. 3B). These masses, combined with using the structure of lacticin 3147 peptides Ltnα and Ltnβ as templates (27), allowed us to predict the structure of the Bli peptides (Fig. 3C). In the case of Bliα, a mass of 3,250 Da is consistent with seven dehydration reactions (i.e., a reduction in the mass of the unmodified peptide, 3,376 Da, by 126 Da). This suggests that only one hydroxy residue remains unmodified. This unmodified residue is most likely to be Ser30, as a corresponding Ser is also unmodified in Bhaα (29). Using the Ltnα structure as a template, it is likely that bridges form between residues 11 and 21, 22 and 27, and 24 and 31. Although a residue corresponding to the remaining cysteine, Cys7, is not present in Ltnα, we predict that this forms a bridge stabilizing the N terminus of the peptide. Bridges involving the most N-terminally located residue are also a feature of Ltnα and Bhaα (29). The mass of Bliβ (3,020 Da) is consistent with the loss of the six most N-terminally located residues, a phenomenon previously associated with Plwα and Bhaα (20, 29), and 12 dehydrations. It is highly likely that bridge formation similar to that observed for Ltnβ (27) is occurring between residues 19 and 23, 25 and 28, and 29 and 32. An additional cysteine, Cys11, which is absent from Ltnβ, also most likely interacts with the N-terminally located residue in a manner analogous to that observed for Bhaα (29).

To confirm that the antimicrobial activity observed was attributable to lichenicidin, we employed a purification method first developed to purify lacticin 3147 from the surface of producing cells. The resulting extract was found to possess antimicrobial activity against indicator strains in well diffusion assays (Fig. 3A for *L. lactis* HP) and was also shown to retain activity when exposed to a variety of different temperatures (42°C to 100°C for 30 min, autoclaving at 121°C for 15 min, and 4°C for 7 days) and to be active over a broad range of pH values (pH 1 to 10) (data not shown). Reverse-phase HPLC analyses of this extract yielded two distinct peaks of mass corresponding to those resulting from CMS (Fig. 3D). Well diffusion assays with HPLC fractions revealed that while each of these possessed antimicrobial activity, as with other two-peptide lantibiotics, synergism was apparent when the individual fractions were combined (Fig. 3D).

DISCUSSION

It has been established that many lantibiotics inhibit clinically relevant pathogens, including multidrug-resistant strains (6, 18, 24, 31, 41). Unsurprisingly, as the potential of these peptides has become more apparent, there have been renewed efforts to identify novel forms with desirable traits. Laboratory-based screening strategies continue to be successful, and re-

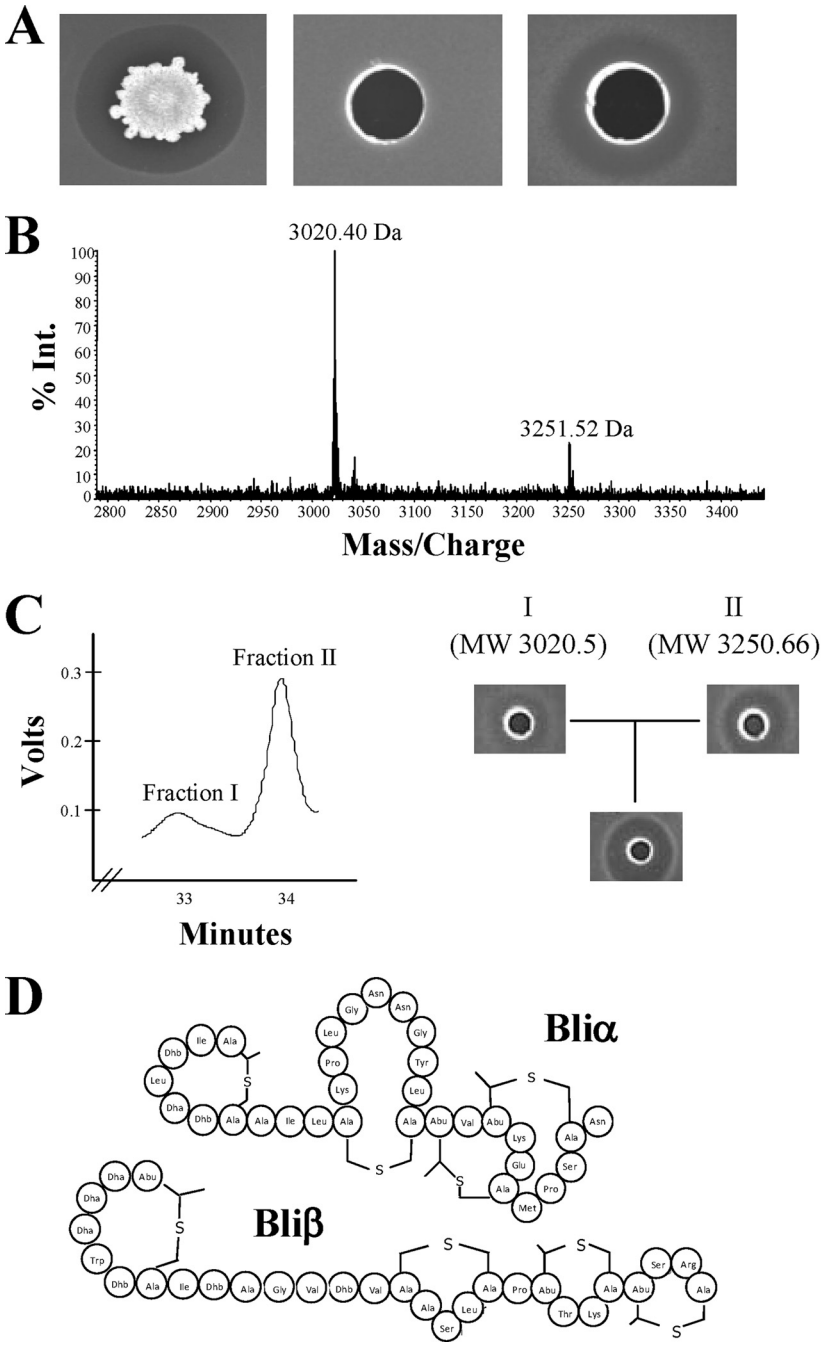

FIG. 3. (A) Deferred antagonism assay and well diffusion assay using *L. lactis* HP as the indicator strain. Cell-free supernatant was added to the well in the center picture. Partially purified lichenicidin was added to the well in the picture on the right. (B) CMS analysis of *B. licheniformis* colonies. Int., intensity. (C) HPLC profile of peptide preparation. Shown are data for mass spectrometry analyses of fractions I and II. A well diffusion assay was performed using *L. lactis* HP as an indicator. MW, molecular weight (in thousands). (D) Predicted structure of the two lichenicidin structural peptides. Structures were predicted using the lacticin 3147 structural peptides Ltnα and Ltnβ as templates (27).

cently, the lantibiotic planosporicin was identified as part of a strategy whereby 120,000 microbial extracts from 40,000 actinomycetes were screened for antimicrobial activity against a staphylococcal indicator strain (6). However, with the wealth of bioinformatic data available in publicly available databases, it is now possible to identify potential lantibiotic producers by using an in silico approach. The approach taken herein relies on the conserved nature of LanM proteins, the biosynthetic enzymes characteristic of group II lantibiotics. This approach was validated by the fact that a large number of LanM proteins, which have already been linked with the production of known lantibiotics, were identified. The additional 61 LanM determinants are located in strains, species (including *Streptococcus pneumoniae*, *B. licheniformis*, *Clostridium beijerinckii*,

and *Clostridium scindens*), genera (*Geobacillus*, *Corynebacterium*, *Bifidobacterium*, *Saccharopolyspora*, and *Salinospora*), and even phyla (the *Cyanobacteria*, i.e., *Anabaena*, *Nostoc*, *Synechococcus*, and *Prochlorococcus*; the *Chloroflexi*, i.e., *Herpetosiphon*; and the *Proteobacteria*, i.e., *Myxococcus*, *Corynebacterium*, *Coxiella*, *Stigmatella*, *Sinorhizobium*, and *Azorhizobium*) that have not previously been associated with lantibiotic production. While it remains to be established if representatives of the *Cyanobacteria*, *Chloroflexi*, or *Proteobacteria* are indeed capable of lantibiotic production, the presence of clusters located in representatives of these phyla is intriguing. Our more detailed investigation of five specific strains (Fig. 1) revealed additional information pertaining to the clusters present in all three of these phyla. A number of cyanobacteria possess *lanM* homologs, including one, *Nostoc punctiforme* PCC 73102, which possesses three such genes. Curiously, the predicted structural peptide encoded within the *A. variabilis* ATCC 29413 plasmid-located cluster more closely resembles peptides encoded within the genomes of the proteobacteria *M. xanthus* and *Stigmatella aurantiaca* and the chloroflexus *H. aurantiacus*, suggesting the existence of mechanisms via which this cluster can become distributed. It may therefore be pertinent that transposases are located in close proximity to the ATCC 29413 cluster and that identified in *G. thermodenitrificans* NG80-2, further highlighting the potentially mobile nature of these clusters. This is consistent with the previous association of many lantibiotic gene clusters with transmissible elements including large conjugative plasmids and transposons.

Another feature of the bioinformatic screen is the number of pathogenic bacteria previously not known to be lantibiotic producers that possess putative LanM determinants, including *Streptococcus pneumoniae*, *Corynebacterium diphtheriae*, *Coxiella burnetii*, and *Clostridium perfringens*. Thus far, cytolysin, produced by enterococci, is the only lantibiotic known to contribute to pathogenicity (8, 12), although lantibiotic production is also a characteristic of selected strains of *Staphylococcus aureus* (32), *Staphylococcus epidermidis* (1, 23), *Streptococcus mutans* (21, 30, 35, 37, 45), and *Streptococcus pyogenes* (22, 44, 47). The addition of the agents capable of causing pneumonia, diphtheria, Q fever, and gastroenteritis to this list provides further evidence that, in addition to researching lantibiotics with a view to targeting these pathogens, there are apparent merits in assessing the contribution of lantibiotic production to the competitiveness, and therefore pathogenicity, of these organisms. One such pathogen, *Streptococcus pneumoniae* SP23-BS72, is potentially the producer of a two-peptide lantibiotic possessing, in addition to the two structural genes, a number of additional genes that are likely to be involved in lantibiotic production. Notably, however, the apparently frameshifted nature of the second LanM determinant may, if genuine, result in one of the two peptides not being modified, which, as a consequence of the synergistic nature of the two peptide lantibiotics, would be predicted to result in the absence of antimicrobial activity (28).

The remaining potentially novel lantibiotic producers were both members of the *Bacillaceae*: *G. thermodenitrificans* NG80-2 and *B. licheniformis* ATCC 14580. Bacilli are the producers of many lantibiotics including subtilin, ericin A, ericin S, mersacidin, sublancin (reviewed in references 46), and haloduracin (25, 29). Both NG80-2 and ATCC 14580 (17, 36) produce a number of enzymes that could have industrial applications. In both cases, a previously unannotated potential structural peptide-encoding gene was identified. While the putative NG80-2 structural peptide shares no sequence similarity with known peptides, bioinformatics revealed that ATCC 14580 was likely to be the producer of a two-peptide lantibiotic. Notably, a detailed bioinformatic analysis was required to identify a second structural peptide-encoding gene in this instance. Similarly, other putative structural peptide determinants, not annotated in the genomes of *G. thermodenitrificans* and *H. aurantiacus*, were revealed. These investigations highlight the merits of performing detailed examinations when what at first appear to be "orphan" LanM determinants, i.e., those apparently unassociated with a corresponding structural determinant, are identified.

As a consequence of the frequent association of bacilli with the production of potent antimicrobials, its nonpathogenic nature, and the likely production of a two-peptide lantibiotic (many of which possess broad-spectrum antimicrobial activity), *B. licheniformis* ATCC 14580 was selected for proof-of-concept studies. HPLC analysis combined with reverse-phase HPLC established that a two-peptide lantibiotic, lichenicidin, is indeed produced. Its activity against *L. monocytogenes*, methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant enterococci suggests that lichenicidin may potentially be used as a clinical therapeutic agent, but further investigations will be required to confirm this.

Although computational analyses described herein and elsewhere cannot be expected to provide a definitive list of all lantibiotic producers, they can identify excellent candidate strains for detailed "wet-lab" experiments. In silico analyses should therefore be considered an attractive alternative to traditional functional assays for the identification of novel lantibiotics.

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